

The type II secretion system (Xcp) of *Pseudomonas putida* is active and involved in the secretion of phosphatases

Florian Putker, Ria Tommassen-van Boxtel,
Michiel Stork, José J. Rodríguez-Herva,
Margot Koster and Jan Tommassen

Summary

The genome of the Gram-negative bacterium *Pseudomonas putida* harbours a complete set of *xcp* genes for a type II protein secretion system (T2SS). This study shows that expression of these genes is induced under inorganic phosphate (P_i) limitation and that the system enables the utilization of various organic phosphate sources. A phosphatase of the PhoX family, previously designated UxpB, was identified, which was produced under low P_i conditions and transported across the cell envelope in an Xcp-dependent manner demonstrating that the *xcp* genes encode an active T2SS. The signal sequence of UxpB contains a twin-arginine translocation (Tat) motif as well as a lipobox, and both processing by leader peptidase II and Tat dependency were experimentally confirmed. Two different *tat* gene clusters were detected in the *P. putida* genome, of which one, named *tat-1*, is located adjacent to the *uxpB* and *xcp* genes. Both Tat systems appeared to be capable of transporting the UxpB protein. However, expression of the *tat-1* genes was strongly induced by low P_i levels, indicating a function of this system in survival during P_i starvation.

Introduction

For many Gram-negative bacteria, the ability to secrete proteins is essential for their survival and growth in

specific environments. Several types of protein secretion mechanisms have been identified (Holland, 2010). One of them, the type II secretion pathway, is found in many bacterial species and is involved in the secretion of a wide range of toxins and hydrolytic enzymes (Filloux, 2004; Cianciotto, 2005). Transport of the exoproteins via this pathway is a two-step process involving a periplasmic intermediate. Most substrates reach the periplasm via the Sec machinery, after which they adopt a folded conformation. Alternatively, the exoproteins are transported in an already folded conformation across the inner membrane via the Tat pathway (Voulhoux *et al.*, 2001). The folded exoproteins are subsequently targeted to the type II secretion system (T2SS) via a still elusive recognition signal, after which the T2SS mediates their transport across the outer membrane.

One of the best studied T2SSs is the Xcp system of *Pseudomonas aeruginosa*, which is involved in the secretion of a large number of different proteins including exotoxin A, lipases, phospholipases C, alkaline phosphatase, chitin-binding protein, and elastase. This system is composed of 12 components, designated XcpA and XcpP-Z (Filloux, 2004). T2SSs share considerable similarities with the machinery required for the assembly of type IV pili. XcpT, XcpU, XcpV, and XcpW show N-terminal sequence similarity with the subunits of type IV pili, and they have been shown to be processed by the same enzyme, XcpA/PilD, that also processes the pilus subunits (Bally *et al.*, 1992). Therefore, they are named pseudopilins, and they are proposed to assemble into a pilus-like structure in the periplasm (Durand *et al.*, 2003). The inner membrane proteins XcpS, XcpY, XcpZ, and the ATPase XcpR are supposedly important for the assembly of this pilus-like structure (Filloux, 2004), while XcpX is another pseudopilin (Bleves *et al.*, 1998) suggested to be involved in its length control (Durand *et al.*, 2005). Upon ATP hydrolysis by XcpR, growth of the pseudopilus may provide the mechanical energy that is required for the passage of the exoproteins through a channel in the outer membrane. This channel is formed by the oligomeric secretin XcpQ (Bitter *et al.*, 1998), which is thought to be linked to the rest of the system via XcpP (Robert *et al.*, 2005).

Much less is known about T2SSs in other *Pseudomonas* species. The presence of *xcp* genes in

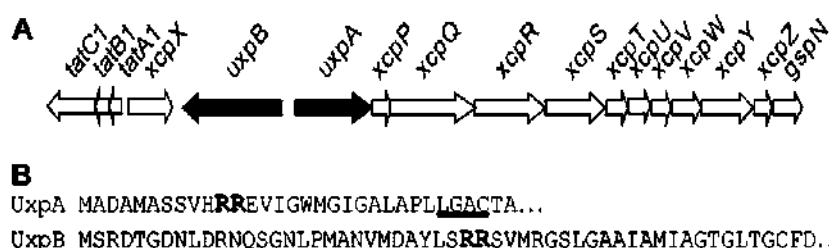


Fig. 1. *In silico* analysis of the T2SS of *P. putida*.

A. Genetic organization of the *xcp*, *uxp* and *tat-1* genes in *P. putida* strain KT2440. Genes are indicated as arrows according to their orientation in the genome. White, black and grey arrows represent the *xcp*, *uxp* and *tat-1* genes respectively.

B. N-termini of the UxpA and UxpB protein precursors of *P. putida* strain WCS358 (de Groot *et al.*, 1996). The twin-arginine motifs (bold) and lipoboxes (underlined) in the signal sequences are indicated.

Pseudomonas putida was initially demonstrated in the plant growth-promoting strain WCS358 in DNA hybridization experiments with the *xcp* genes of *P. aeruginosa* as probes (de Groot *et al.*, 1991). Subsequent sequence analysis identified the *xcpP-Z* genes all oriented in the same direction in what appears to be one single operon (Fig. 1A) (de Groot *et al.*, 1996; 1999), while the *xcpA/pilD* gene, like in *P. aeruginosa*, was located separately within a cluster of genes involved in type IV pilus biogenesis (de Groot *et al.*, 1994). The *xcpP-Z* operon also contains a gene encoding a GspN homologue (Fig. 1A), a component of the T2SS in some bacteria, but not in others including *P. aeruginosa*. However, an *xcpX* gene was not found in the operon (de Groot *et al.*, 1996; 1999). This, along with the observation that inactivation of *xcp* genes did not alter the extracellular protein profile (de Groot *et al.*, 1996), suggested that the Xcp system in *P. putida* might be inactive.

Since these initial studies, the genome sequences of several *P. putida* strains, including strain KT2440 (Nelson *et al.*, 2002), have been unravelled. When we searched these genome sequences, we found the missing *xcpX* homologue (locus tag PP1042 in the genome sequence of KT2440; expect 4e-15) upstream of the operon containing *xcpP-Z* (Fig. 1A). This observation suggested the presence of a complete Xcp system in *P. putida* and encouraged us to re-investigate whether this T2SS is indeed active in the secretion of proteins.

Results

UxpA and UxpB, two potential substrates of the Xcp system

Upstream of *xcpP*, with overlapping stop and start codon, a gene was found named *uxpA* (upstream of *xcpP* A) potentially encoding a protein of 550 amino-acid residues (Fig. 1A) (de Groot *et al.*, 1996). This putative protein contains an N-terminal signal sequence ending with a lipobox motif (underlined in Fig. 1B) suggesting that it is processed into a lipoprotein by the subsequent action of the pro-lipoprotein modification enzymes Lgt, the lipoprotein signal peptidase (Lsp or leader peptidase II) and Lnt

(Okuda and Tokuda, 2011). The position of this gene in the same operon as *xcpP-Z* and the presence of the signal sequence suggested that this protein might be a substrate for the Xcp system (de Groot *et al.*, 1996). Since no UxpA homologues of known function had been identified, we performed a conserved-domain search at NCBI and found that the protein belongs to the metallophosphatase (MPP) superfamily (Fig. S1), which includes 5' nucleotidases, phosphodiesterases and phosphoprotein phosphatases, suggesting that UxpA might be involved in phosphate acquisition.

Upstream of *uxpA*, a partial open reading frame in the opposite orientation, named *uxpB*, was detected (de Groot *et al.*, 1996). Interestingly, the protein putatively encoded by this gene also contains an N-terminal signal sequence ending with a lipobox (Fig. 1B). This and the position of *uxpB* in between *xcpX* and the *uxpA-xcpZ* operon (Fig. 1A) suggest that also the UxpB protein may be a substrate of the Xcp machinery. Conserved domain searches with the complete 691-residue UxpB protein of *P. putida* strain KT2440 (locus tag PP_1043) revealed that the corresponding gene putatively encodes an alkaline phosphatase of the PhoX family (Fig. S1).

Several PhoX-family members, including those of *Pseudomonas fluorescens* Pf0-1 (Monds *et al.*, 2006) and *Campylobacter jejuni* (van Mourik *et al.*, 2008), have been reported to be exported from the cytoplasm via the Tat system. Also the signal sequence of the UxpB protein contains a twin-arginine motif (bold in Fig. 1B), indicating that it might be exported via the Tat pathway as well. Interestingly, also the signal sequence of UxpA contains a twin-arginine motif (Fig. 1B). A complete Tat export system usually consists of the products of three genes, *tatA-C* (Palmer *et al.*, 2005). We identified two complete sets of *tat* genes in the genome sequence of KT2440 with sequence identities between the TatA, TatB and TatC components of 36%, 33% and 61% respectively. Interestingly, one of these sets of *tat* genes is located directly adjacent to *xcpX* (Fig. 1A), while the other is located elsewhere on the chromosome (locus tags PP_5016-5018). This suggests that the proteins encoded by the former set of genes, here designated *tatA1*, *tatB1*, and *tatC1* (locus

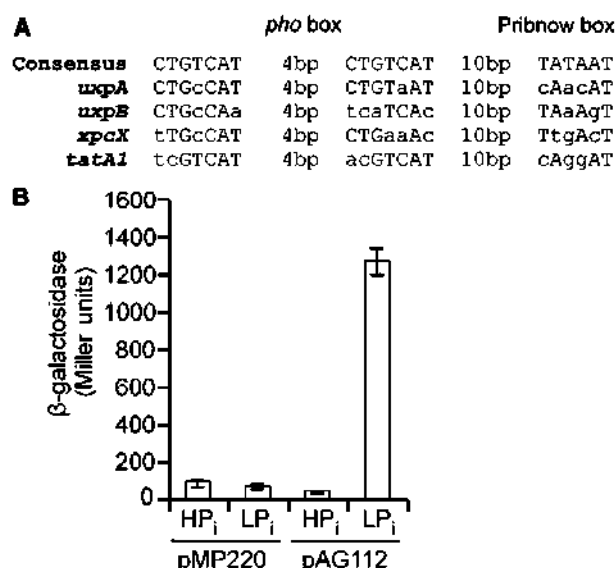


Fig. 2. Phosphate regulation of the expression of the *uxp*, *xcp* and *tat-1* genes.

A. Putative *pho* boxes in the promoter regions of *uxpA*, *uxpB*, *xcpX* and *tatA1*. Nucleotides complying with the consensus sequence are capitalized.

B. Induction of the *uxpA* promoter in response to P_i availability. Cells of strain KT2440 carrying either the empty promoter-probe vector pMP220 or pAG112, which contains the *uxpA* promoter inserted upstream of the promoter-less *lacZ* gene in pMP220, were grown overnight in P_i -limiting medium either supplemented (HP_i) or not (LP_i) with 10 mM KH_2PO_4 . Promoter activity was determined by measuring the β -galactosidase activity. Bars represent the averages of three independent measurements, and standard deviations are indicated.

tags PP_1041, PP_1040, and PP_1039 respectively), might specifically be dedicated to the export of the Xcp substrates UxpA and UxpB.

Together, our *in silico* analysis suggests that the *P. putida* genome contains a complete set of *xcp* genes that would participate in the secretion of enzymes involved in phosphate acquisition. Furthermore, it also suggests that these enzymes could be exported from the cytoplasm via the Tat pathway and, at the periplasmic side of the inner membrane, may be modified into lipoproteins before being secreted via the Xcp system into the extracellular milieu.

P_i regulation of *xcp*, *uxp* and *tat* genes

If the Xcp system and its substrates are involved in phosphate acquisition, the expression of the corresponding genes might be regulated by P_i availability. In *Escherichia coli*, P_i -regulated gene expression requires a two-component regulatory system consisting of PhoB as the response regulator and PhoR as the histidine kinase (Wanner, 1993). A similar system is operative in *P. aeruginosa* (Filloux *et al.*, 1988), and genes homologous to

phoB and *phoR* can also be identified in the *P. putida* KT2440 genome sequence (locus tags PP_5320 and PP_5321 respectively). In *E. coli*, genes that are activated by phosphorylated PhoB under P_i limitation contain at least one copy of a *pho* box in the promoter region, which consists of two heptameric repeats with the consensus sequence CTGTCAT, separated by an AT-rich 4 bp sequence (Wanner, 1993). This *pho* box is located 10 bp upstream of the Pribnow box. As an initial approach to determine whether the *xcp* system of *P. putida* is regulated by P_i availability, we searched the promoter regions for putative *pho* boxes. Indeed, potential *pho* boxes with appropriately spaced putative Pribnow boxes were found in the promoter regions upstream of *uxpA*, *uxpB*, *xcpX*, and *tatA1* (Fig. 2A).

The putative P_i -regulated expression of the *uxp*, *xcp* and *tat* genes was then experimentally assessed by quantitative real-time reverse-transcription PCR (qRT-PCR). The relative transcript levels of the *uxpA*, *uxpB*, *xcpX*, *xcpQ*, and *tatA1* genes were induced 7.6 ± 5.3 , 85.4 ± 6.0 , 3.2 ± 1.8 , 95.4 ± 29.4 , and 18.5 ± 5.8 fold respectively, by growth in low- P_i medium compared with high- P_i medium. In contrast, expression of the *tatA2* gene was not affected by P_i limitation. These results show that expression of *uxpA* and *uxpB* as well as that of the genes putatively encoding the secretion machinery is induced under P_i limitation.

While the qRT-PCR experiments showed that expression of *uxpB* is strongly affected by P_i availability, *uxpA* expression was more moderately affected. To verify the P_i -regulated expression of *uxpA*, plasmid pAG112, which carries the *uxpA* promoter region inserted upstream of the promoterless *lacZ* gene in the promoter probe vector pMP220, was introduced into strain KT2440. Growth of the resulting strain under P_i limitation resulted in an approximately 30-fold increase in β -galactosidase activity when compared with growth under high P_i conditions (Fig. 2B). In a strain carrying the empty promoter-probe vector, β -galactosidase activity was not affected by P_i availability (Fig. 2B). Similar results were obtained when the plasmids were introduced into strain WCS358 (data not shown). These results confirm the regulation of the *uxpA* promoter by P_i availability. Further evidence for the P_i -regulated expression of *uxpB* was obtained after identification of the gene product (see below).

Role of the Xcp system in phosphate acquisition

To study the possible role of the T2SS of *P. putida* in phosphate acquisition, we compared the growth of the wild-type strain KT2440 and its *xcpS* and *xcpX* mutant derivatives KT Δ *xcpS::gm* and KT Δ *xcpX::km* respectively, on different phosphate sources. The bacteria were inoculated on P_i -free synthetic-medium plates, on which filter

discs with the different phosphate sources were placed. The wild-type strain grew well around the discs with glucose 6-phosphate, 5'-AMP, the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP), and, albeit poorly, UDP-glucose (Fig. 3). The mutants were not capable of growing on 5'-AMP, UDP-glucose and XP as sole phosphate sources, while growth on glucose 6-phosphate appeared reduced (Fig. 3). Growth of all strains was equally well supported around filter discs containing P_i (Fig. 3). Similar results were obtained when the experiment was performed with *P. putida* strain WCS358 and its $\Delta xcpRST$ derivative CE1430 (data not shown). Thus, these results confirm the postulated role of the Xcp system in the secretion of enzymes involved in phosphate acquisition.

UxpB is a phosphatase

The observation that the wild-type *P. putida* strains can utilize various organic phosphate sources (Fig. 3) indicates that they produce (a) phosphatase(s). To identify such phosphatase(s), we developed a zymography-based assay. Proteins from whole cell extracts and culture supernatants of cells grown under high- P_i or low- P_i conditions were separated by semi-native SDS-PAGE, and the gels were subsequently incubated with the chromogenic phosphatase substrate XP. One phosphatase with an apparent molecular weight of ~55 kDa was clearly detected on the zymograms in the cell lysates of strains KT2440 (Fig. 4A, lane 3) and WCS358 (data not shown) grown under P_i limitation.

Even though the apparent molecular weight of the phosphatase detected on the zymograms is considerably lower than the calculated molecular mass of UxpB, i.e. 74.3 kDa for the precursor and ~70 kDa (depending on the length of the acyl chains) for the mature form, we considered the possibility that it represents UxpB, since UxpB is a PhoX-family member, which should be able to hydrolyse XP. To verify that the identified phosphatase on the zymograms was indeed UxpB, a recombinant gene encoding the UxpB protein of strain KT2440 with a C-terminal His tag was cloned on pBBR1MCS-5. Strain KT2440 carrying the resulting construct produced the phosphatase even in the presence of high P_i concentrations (Fig. 4A, lane 6). The recombinant enzyme migrated at the same position as the identified phosphatase in the wild-type strain, and immunoblotting with antibodies directed against the His tag indeed reacted with a protein in this position (Fig. 4B, lane 2). However, the antibody reacted also with two bands with apparent molecular weights of ~80 kDa. Upon denaturation of the samples by heating in the presence of dithiothreitol (DTT), the faster migrating protein was completely lost, but the double band at ~80 kDa was clearly detected (Fig. 4B, lane 3).

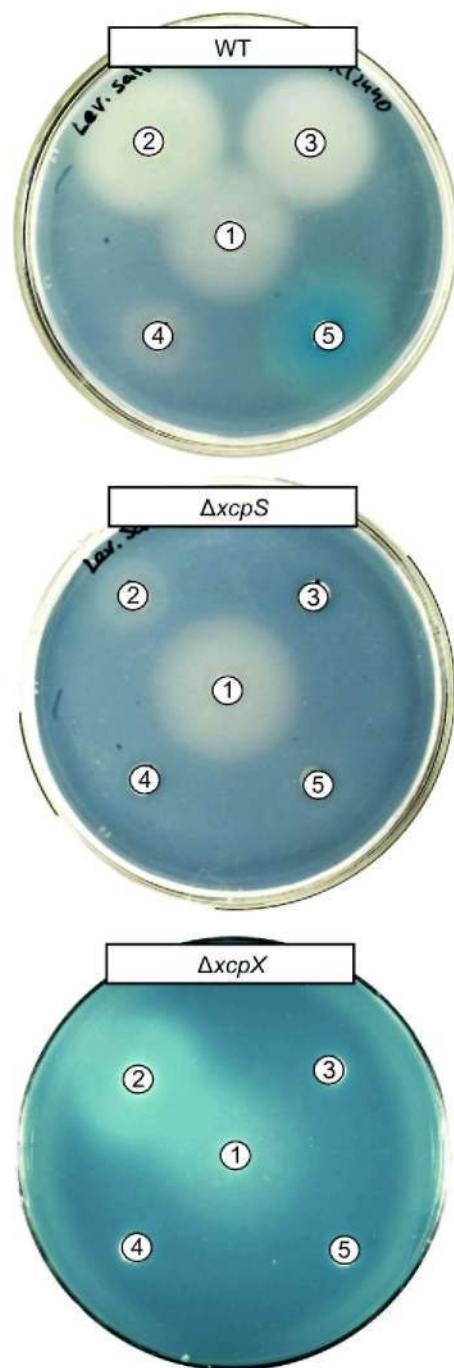


Fig. 3. Role of the Xcp system in phosphate acquisition. Utilization of different phosphate sources by wild-type strain KT2440 (WT) and its isogenic *xcpS* mutant derivative $KT\Delta xcpS::gm$ and *xcpX* mutant derivative $KT\Delta xcpX::km$ was determined. Cells were applied in top layers on synthetic medium plates lacking any phosphate source. Filter discs with various phosphate sources were placed on top of the plates. 1, KH_2PO_4 ; 2, glucose 6-phosphate; 3, 5'-AMP; 4, UDP-glucose; 5, XP. Growth around the filters was assessed after 2 days of incubation at 30°C.

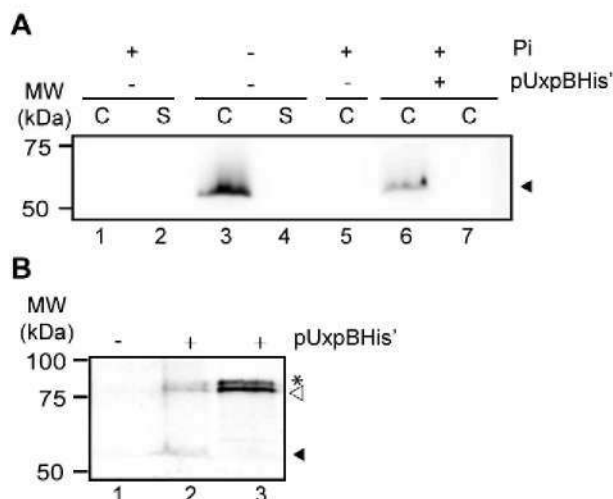


Fig. 4. UxpB of *P. putida* is a phosphatase. A. Strain KT2440 and a derivative expressing a C-terminally His-tagged UxpB protein from plasmid pUxpBHis' were grown overnight in low-phosphate medium either supplemented or not with 10 mM KH_2PO_4 as indicated. Cells (C) and supernatants (S) were separated and analysed by semi-native SDS-PAGE and zymography using XP as the substrate. The sample in lane 7 was denatured by heating for 5 min at 100°C in sample buffer containing a reducing agent (DTT) prior to SDS-PAGE. The arrowhead indicates the phosphatase band detected; the positions of molecular-mass marker proteins are indicated (in kDa) at the left. B. The same samples as in lanes 5–7 of panel A were blotted and immunodetection was performed using anti-His antibodies. The molecular weight markers (left) and the precursor (asterisk), denatured mature (open arrowhead) and folded forms (closed arrowhead) of His-tagged UxpB are indicated.

Presumably, these bands correspond to the denatured mature form of the protein and to the precursor, which might accumulate due to overexpression from the plasmid and/or because the *tat-1* genes, which putatively encode the transport system for the translocation of UxpB across

the inner membrane, are poorly expressed at high P_i concentrations. As expected, the phosphatase activity on zymograms was completely lost under denaturing conditions (Fig. 4A, lane 7). These results show the existence of the recombinant UxpB in three forms of which the 55 kDa form is the folded and active protein, while the slower migrating ones represent the denatured mature form and the precursor of the protein.

UxpB is a lipoprotein

Since UxpB could readily be detected, we further focused on this protein as a model to unravel the secretion route of the Xcp substrates in *P. putida*. Under our standard culture conditions, the enzyme was found associated with the cells and not in the extracellular medium (Fig. 4A). This result could be explained by UxpB being a lipoprotein as suggested by the presence of a lipobox in the signal sequence (Fig. 1B). Thus, after secretion via the T2SS, the protein may remain attached to the bacterial outer membrane via its lipid anchor. Lipoproteins are processed by leader peptidase II, an enzyme that is specifically inhibited by globomycin (Inukai *et al.*, 1978). To investigate whether UxpB is indeed a lipoprotein, globomycin was added to KT2440 cells expressing His-tagged UxpB. Phosphatase activity was considerably lower after growth for 3.5 h in the presence of globomycin relative to cells grown in the absence of the antibiotic (Fig. 5A). In addition, immunoblotting revealed the accumulation of the UxpB precursor under these conditions (Fig. 5B), showing that UxpB is indeed a substrate for leader peptidase II.

UxpB is a substrate of the Xcp system

Based on the results obtained so far, we hypothesized that UxpB is transported via the Xcp system to the cell

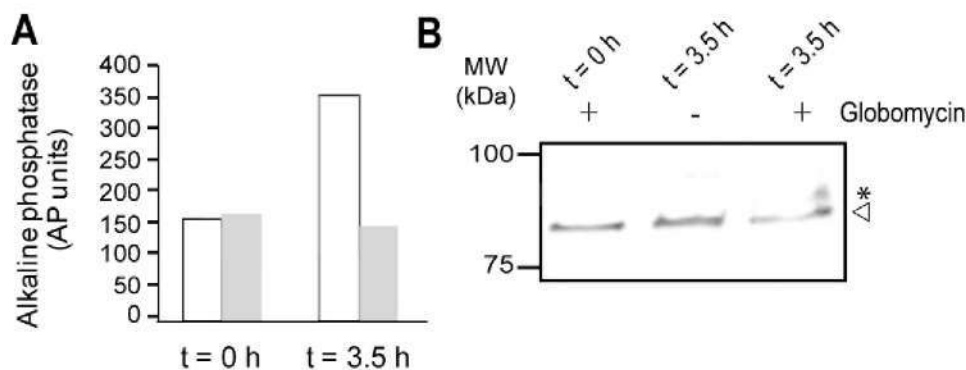


Fig. 5. Globomycin inhibits the processing of UxpB.

A. To exponentially growing cells of strain KT2440 producing His-tagged UxpB from pUxpBHis' in low- P_i medium either no globomycin (white bars) or 120 $\mu\text{g ml}^{-1}$ globomycin (grey bars) was added at $t = 0$ after which growth was continued for 3.5 h and alkaline phosphatase activity was determined.

B. Samples of the cells grown in the presence or absence of globomycin and taken at the time points indicated were analysed by SDS-PAGE and immunoblotting using an antiserum directed against the His-tag. The positions of molecular weight markers are indicated at the left, and those of mature UxpB and the UxpB precursor are indicated with an asterisk and an arrowhead, respectively, at the right.

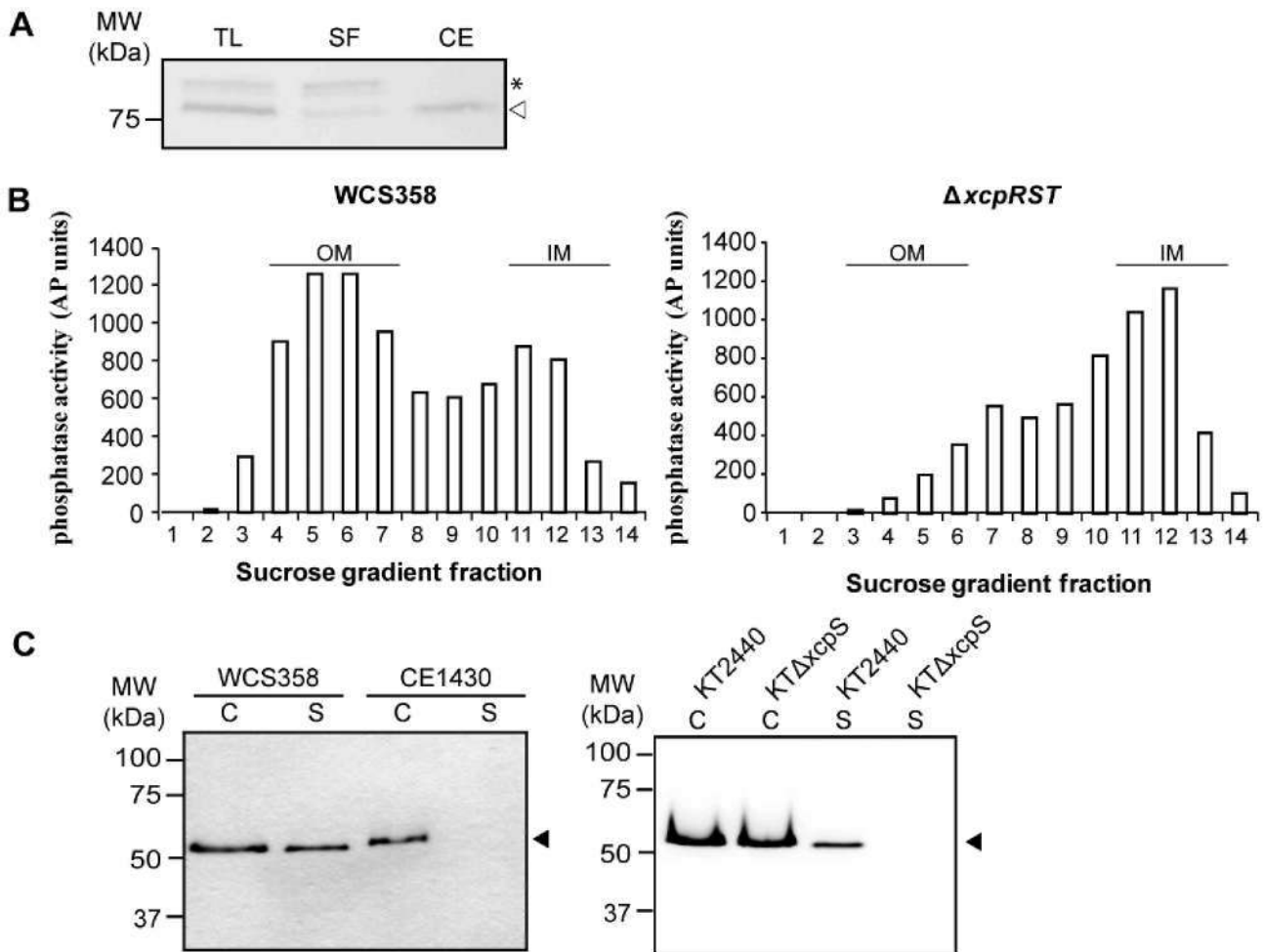


Fig. 6. UxpB is an Xcp substrate.

A. KT2440 cells producing His-tagged UxpB from plasmid pUxpBHis⁺ were grown under P_i limitation. The cells were disrupted by sonication and cell envelopes (CE) and soluble fraction (SF) were separated by centrifugation and analysed by denaturing SDS-PAGE. TL, total cell lysate. The precursor and processed mature forms of UxpB are indicated with an asterisk and an open arrowhead respectively.

B. Membranes of wild-type strain WCS358 and of the $\Delta xcpRST$ mutant CE1430 were isolated and separated by sucrose gradient centrifugation, and the phosphatase activities in the fractions were determined. The fractions representing the inner membranes and the outer membranes were identified based on the NADH-oxidase activity and the presence of OprF, respectively (results not shown), and are indicated.

C. Strain WCS358 and its $\Delta xcpRST$ mutant CE1430 (left panel) and strain KT2440 and its $xcpS$ mutant derivative KT $\Delta xcpS$ (right panel) were grown under P_i limitation with low aeration. Cells (C) and culture supernatant (S) were analysed by zymography after semi-native SDS-PAGE. The molecular weight marker (left) and UxpB (arrowhead) are indicated.

surface where it remains anchored to the outer membrane via its lipid moiety. To verify the subcellular location of UxpB, cells expressing His-tagged UxpB were fractionated. As expected, the processed mature form of the protein fractionated mainly with the membranes consistent with its lipoprotein nature, while the precursor form was detected exclusively in the soluble fraction (Fig. 6A).

To determine in which membrane the mature form of UxpB is localized, the inner and outer membranes of strain WCS358 were separated by sucrose gradient density centrifugation. The highest phosphatase activity was detected in the outer-membrane fractions (Fig. 6B,

left panel). Analysis of the fractions by SDS-PAGE and zymography confirmed the presence of UxpB predominantly in the outer-membrane fractions (data not shown).

Next, we determined whether the outer-membrane localization of the phosphatase is dependent on the Xcp system. To this end, we fractionated cells of strain CE1430, a $\Delta xcpRST$ mutant derivative of WCS358. After separation of inner and outer membranes, phosphatase activity was found associated with the inner-membrane fractions (Fig. 6B, right panel). These experiments demonstrate that transport of the phosphatase to the outer membrane is dependent on the Xcp system.

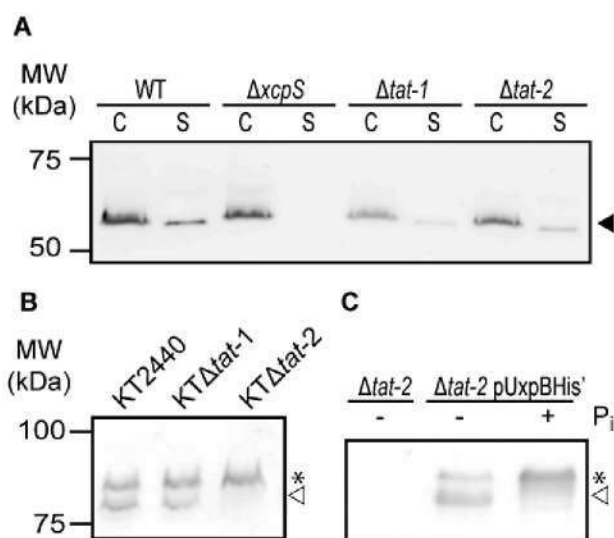


Fig. 7. Tat-dependency of UxpB secretion.

A. Strain KT2440 (WT) and its derivatives KT $\Delta xcpS$, KT $\Delta tat-1$ and KT $\Delta tat-2$ were grown under P_i -limiting conditions and low aeration, and equal amounts of cells (C) and culture supernatant (S) were analysed by zymography after semi-native SDS-PAGE. The positions of molecular weight markers (left) and UxpB (closed arrowhead) are indicated.

B. Strain KT2440 and its derivatives KT $\Delta tat-1$ and KT $\Delta tat-2$, all producing His-tagged UxpB from pUxpBHis⁺, were grown in medium supplemented with P_i . Proteins of whole cell lysates were separated by denaturing SDS-PAGE and analysed by immunoblotting with an antibody recognizing the His-tag. The precursor and processed mature forms of UxpB are indicated with an asterisk and an open arrowhead respectively.

C. Strain KT $\Delta tat-2$ and its derivative producing His-tagged UxpB from pUxpBHis⁺ grown overnight in low- P_i medium with 0.05% trypticase peptone were inoculated in the same medium supplemented or not with 10 mM KH_2PO_4 as indicated and grown at 30°C with aeration (200 r.p.m.). Proteins of whole cell lysates were separated by denaturing SDS-PAGE and analysed by immunoblotting with an antibody recognizing the His-tag. The precursor and processed mature forms of UxpB are indicated with an asterisk and an open arrowhead respectively.

The substrates of T2SSs of various bacteria are usually not substituted with a lipid moiety. One of the rare examples is the substrate of the archetype T2SS, the Pul system of *Klebsiella oxytoca*. The substrate of this system, pullulanase, remains at least temporarily bound to the bacterial cell surface before it is released into the extracellular environment (d'Enfert *et al.*, 1987). In contrast, under our standard growth conditions, UxpB was found exclusively associated with the cells and not to be released into the extracellular medium (Fig. 4A). Hence, we searched for growth conditions that would favour the release of UxpB. We observed that a considerable amount of UxpB was released into the extracellular medium of both strains WCS358 and KT2440 when the bacteria were grown with lower aeration (shaking at 50 r.p.m.) (Fig. 6C) than in our routine culture conditions (shaking at 200 r.p.m.). Secretion of the phosphatase was completely abolished in the $\Delta xcpRST$ mutant CE1430 of

strain WCS358 and the $xcpS$ mutant derivative KT $\Delta xcpS$ of KT2440 (Fig. 6C). Together, it can be concluded that the transport of the UxpB phosphatase to the outer membrane and its release from the cell surface are dependent on a functional Xcp system.

UxpB is a Tat substrate

The signal sequences of UxpA and UxpB contain the typical twin-arginine motif for proteins transported via the Tat pathway (Fig. 1B), and one of the two *tat* operons present in the *P. putida* KT2440 genome is located directly adjacent to the *xcp/uxp* genes (Fig. 1A) and induced under P_i limitation as described above. Therefore, we assumed that the products of this *tat-1* operon are dedicated to the transport of UxpA and UxpB. To investigate the possible role of the Tat-1 and Tat-2 systems in UxpB transport, appropriate mutants were constructed, and the secretion of UxpB was investigated under low- P_i and low-aeration conditions. Neither mutation abolished the production and secretion of the UxpB protein (Fig. 7A). Thus, either UxpB is not a Tat substrate or the two Tat systems have redundant activities.

To discriminate between the possibilities postulated above, we attempted to inactivate both systems in a single strain, which unfortunately failed. As an alternative approach, we made use of the observation that the *tat-1* system is regulated by P_i availability and is, therefore, expected to be poorly or not expressed in high- P_i medium. Hence, we decided to evaluate the processing of His-tagged UxpB produced constitutively from plasmid pUxpBHis⁺ in strain KT2440 and its isogenic Δtat mutant derivatives after growth in high- P_i medium. Immunoblotting revealed under these conditions the presence of both the precursor and the mature form of the His-tagged UxpB after its production in the wild-type strain as well as in the *tat-1* mutant (Fig. 7B). However, in the $\Delta tat-2$ mutant, processing of His-tagged UxpB into the mature form was totally abolished (Fig. 7B). This result demonstrates that export of UxpB from the cytoplasm is dependent on the Tat-2 system under high P_i conditions when the Tat-1 system is poorly expressed. Then, we tested whether UxpB could be exported in the $\Delta tat-2$ mutant under low- P_i conditions when the Tat-1 system is expressed, which indeed appeared to be the case (Fig. 7C). Together, these results demonstrate that UxpB is indeed a Tat substrate that is exported when either one of the Tat systems is expressed.

The two P. putida Tat systems are functionally redundant

The results presented above indicated that the two Tat systems of *P. putida* have redundant activities, at least

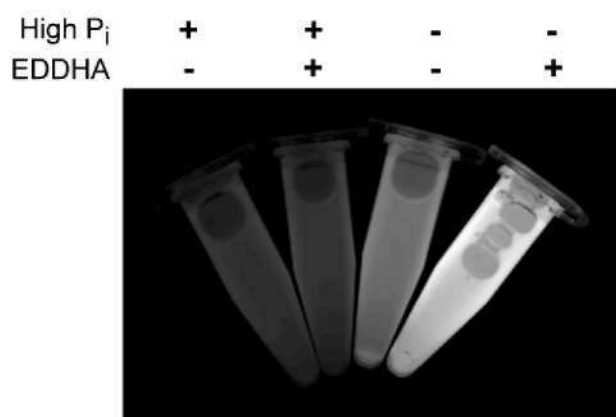


Fig. 8. Production of pyoverdine in a $\Delta tat-2$ mutant is restored after induction of *tat-1* under P_i limitation. Cells from an overnight culture of strain KT $\Delta tat-2$ in LB were used to inoculate P_i-limiting medium either supplemented or not with 10 mM KH₂PO₄ as indicated. The cultures were grown at 30°C with aeration (200 r.p.m.). At an OD₆₀₀ of ~ 1.4, when P_i limitation was reached in the low-P_i cultures as verified in alkaline-phosphatase activity assays, EDDHA was either added or not and growth was continued until an OD₆₀₀ of ~ 3.2 was reached, and fluorescence was visualized in UV light.

with respect to UxpB. To gain more insight in the overlapping specificities of the two systems, another Tat substrate of *P. putida* was identified. A Δtat mutant of *P. aeruginosa* is unable to transport the PvdN protein, which is essential for the synthesis of the fluorescent siderophore pyoverdine, to the periplasm (Voulhoux *et al.*, 2006). A gene encoding a PvdN homologue is present in the *P. putida* genome (locus tag PP_4214) and its putative product contains a signal sequence with a twin-arginine motif. To study the role of the two Tat systems in siderophore production, strain KT2440 and the two Δtat mutant derivatives were grown in high-P_i medium in the presence of the iron chelator ethylenediamine-di- α -hydroxyphenylacetic acid (EDDHA) and fluorescence was assessed upon exposure to UV light. The wild-type and $\Delta tat-1$ mutant produced similar levels of the fluorescent siderophore (results not shown). In contrast, the $\Delta tat-2$ mutant did not produce fluorescence (Fig. 8), showing that, like in *P. aeruginosa*, siderophore biosynthesis is dependent on a functional Tat system. When the $\Delta tat-2$ mutant was grown under P_i limitation prior to the addition of EDDHA, siderophore production was restored (Fig. 8), although not to wild-type levels. This result shows that the Tat-1 system, whose expression is induced under P_i limitation, can functionally substitute for the Tat-2 system, at least partially. In conclusion, although the two Tat systems of *P. putida* show differentially regulated expression, they display overlapping substrate specificities.

Discussion

The T2SS system encoded by the *xcp* genes of *P. putida* was previously considered to be inactive because a

homologue of the *xcpX* gene was not found in the *xcp* gene cluster and because no differences in the extracellular protein profiles of the wild-type strain WCS358 and an *xcp* mutant were detected (de Groot *et al.*, 1996; 1999). We demonstrate here that this T2SS is complete and active in the secretion of (a) phosphatase(s) under P_i-limiting growth conditions. The previous failure to detect changes in the extracellular protein profile of the *xcp* mutant can be explained by the observations that (i) the expression of the system is repressed under high-P_i conditions that were then applied, and (ii) the substrates of the system are lipoproteins that remain attached to the cell surface when the cultures are normally aerated as demonstrated for UxpB and presumably is also the case for UxpA. Actually, we previously detected alkaline phosphatase activity after growth of strain WCS358 under P_i limitation (de Groot *et al.*, 1991), but the observation that this activity was associated with the cells and not detected in the extracellular medium, led to the erroneous conclusion that this enzyme was not an Xcp substrate. It is interesting to note that *P. aeruginosa* contains, besides the Xcp system, a second T2SS, which is involved in the secretion of an alkaline phosphatase, LapA, under P_i limitation (Ball *et al.*, 2002). This T2SS, called the Hxc system, is not particularly related to the Xcp system of *P. putida* in that the genetic organization of the *hxc* genes is completely different and the LapA protein is not homologous to UxpA or UxpB. Also *P. putida* has a second T2SS, the Xcm system, which is involved in the secretion of a manganese oxidase (de Vrind *et al.*, 2003). This Xcm system is very deviant from the regular T2SSs, since homologues of XcpP, XcpY and XcpZ were not found and because the system contains, instead of homologues of the four pseudopilins XcpT, XcpU, XcpV, and XcpW, only three pseudopilins, which are all homologous to XcpT.

The signal sequences of UxpA and UxpB (Fig. 1B) suggested that both proteins are exported from the cytoplasm via the Tat system and modified by the lipoprotein-modification enzymes before being secreted via the T2SS. Experimental evidence for this secretion and modification pathway could indeed be provided for UxpB. Thus, the Xcp-mediated type II secretion pathway of *P. putida* appears to combine various rare characteristics: (i) T2SS substrates usually deploy the Sec pathway, rather than the Tat pathway for export from the cytoplasm, but exceptions have previously been reported (e.g. Voulhoux *et al.*, 2001), (ii) T2SS substrates are commonly not modified with a lipid moiety, although exceptions have been reported (e.g. d'Enfert *et al.*, 1987), and (iii) although Tat-dependent lipoproteins have been reported, for example in the archaeon *Haloferax volcanii* (Giménez *et al.*, 2007) and in the Gram-positive bacterium *Streptomyces coelicolor* (Thompson *et al.*, 2010), their occurrence in Gram-negative bacteria appears exceptional. In

addition, although reported before in Gram-positive bacteria (Jongbloed *et al.*, 2004), *P. putida* is, to our knowledge, the first reported Gram-negative bacterium containing two functional Tat translocases.

The secretion pathway and the final localization of UxpB are somewhat reminiscent of those of the pectin lyase homologue PnlH of *Dickeya dadantii*. Like UxpB, PnlH is exported from the cytoplasm via the Tat system before it is transported to the bacterial cell surface via a T2SS. However, unlike UxpB, PnlH is not a lipoprotein but it remains anchored in the outer membrane via its uncleaved Tat signal sequence (Ferrandez and Condemine, 2008).

UxpB is a member of the PhoX family of alkaline phosphatases. Previously, an UxpB homologue of *P. fluorescens* Pf0-1 (locus tag pfl5179; expect 1e-120) was reported to be exported via the Tat pathway (Monds *et al.*, 2006). However, in contrast to UxpB of *P. putida*, this PhoX of *P. fluorescens* is not a lipoprotein and, consistent with the absence of recognizable homologues of *xcp* genes in the available *P. fluorescens* genome sequences, PhoX remains periplasmic. Interestingly, a homologue of *uxpB/phoX* can also be identified in the genome sequence of *P. aeruginosa* PAO1 (locus tag PA2635; expect 7e-176). To our knowledge, the protein encoded by this gene has not been studied so far. Like PhoX of *P. fluorescens*, the signal sequence of this protein shows a twin-arginine motif but no lipobox. It will be interesting to determine whether the PA2635-encoded protein, in spite of the absence of a lipid moiety, is secreted via either one of the two T2SSs of *P. aeruginosa*. In this respect, it should be noted that lipid modification of pullulanase of *K. oxytoca*, one of the rare examples of a lipoprotein secreted via a T2SS, appeared not to be essential for secretion of the protein into the extracellular medium (Poquet *et al.*, 1993). It is also interesting to note that our BLAST searches identified PhoX-family members more closely related to UxpB of *P. putida* than those of *P. fluorescens* (39% amino-acid sequence identity, 51% similarity) or *P. aeruginosa* (44% sequence identity, 56% similarity) in more distantly related bacterial species, such as *Shewanella frigidimarina* NCIMB 400 (locus tag ABI70469.1; 60% sequence identity, 72% similarity) and *Vibrio alginolyticus* (locus tag VMC_10620; 59% sequence identity, 71% similarity). This suggests that the *phoX* gene can be spread among bacterial species by horizontal gene transfer. Like in *P. fluorescens* and *P. aeruginosa*, the *phoX* genes in *S. frigidimarina* and *V. alginolyticus* are not located on the chromosome in a cluster of genes encoding a T2SS.

For the producing cell, it is obviously advantageous to retain secreted hydrolytic enzymes at the cell surface. The products of hydrolysis are generated close to the cell surface and can readily be taken up by producing cells. In

contrast, entire bacterial communities may profit from the activity of enzymes that are released in the environment. Thus, planktonic cells may prefer to retain hydrolytic enzymes at the cell surface, while bacteria living in biofilms may release such enzymes to the benefit of the entire community. In this respect, it is interesting to note that P₂ limitation, i.e. the condition that leads to the production and secretion of UxpA and UxpB in *P. putida*, has been reported in several *Pseudomonas* species to induce the dispersal of biofilms (Monds *et al.*, 2001; 2007), thus leading to a life style in which it is preferable to retain the enzymes at the cell surface. Furthermore, we noticed considerable release of UxpB from the cells when aeration was reduced. Such low oxygen tension may mimic the conditions in a biofilm. The molecular mechanism that leads to the release of the cell-surface-exposed enzymes still needs to be investigated.

In the absence of a functional Xcp machinery, UxpB was found to be retained at the inner membrane (Fig. 6B). Usually, lipoproteins are transported through the periplasm to the inner leaflet of the outer membrane via the Lol system, unless they contain a Lol-avoidance (or inner-membrane-retention) signal (Okuda and Tokuda, 2011). In *E. coli*, the presence of an aspartate residue at the +2 position, i.e. adjacent to the acylated cysteine, usually functions as Lol-avoidance signal (Yamaguchi *et al.*, 1988), while the residue present at the +3 position can contribute to the strength of the signal (Gennity and Inouye, 1991; Terada *et al.*, 2001). Pullulanase of *K. oxytoca* contains an Asp at the +2 position and its substitution by other residues infringed efficient secretion via the T2SS (Pugsley and Kornacker, 1991) demonstrating that retention at the cytoplasmic membrane is important for the lipoprotein to be recognized by the T2SS. Also UxpB appears to possess a Lol avoidance signal, since it was not sorted to the outer membrane in the absence of a functional T2SS (Fig. 6B). However, neither UxpB nor UxpA contains an Asp at the +2 position (Fig. 1B). The sorting rules for lipoproteins in *P. aeruginosa* appear to be different from those identified in *E. coli*. Although Asp⁺² is functional as a Lol-avoidance motif, also Lys⁺³Ser⁺⁴ appeared to constitute an effective Lol-avoidance motif in *P. aeruginosa* (Narita and Tokuda, 2007) and additional signals have been identified (Lewenza *et al.*, 2008). However, neither of these signals is present in UxpA or UxpB. In *E. coli*, also other amino acids (aromatic residues, proline, glycine) can function as Lol-avoidance signals when engineered at the +2 position (Seydel *et al.*, 1999), but they are never found in *E. coli* lipoproteins. UxpB does contain a Phe at +2 (Fig. 1B) and it is likely that this residue, possibly in combination with the Asp at +3, functions as a Lol avoidance motif in *P. putida*. In UxpA, no Lol-avoidance motif similar to those previously identified in *E. coli* or *P. aeruginosa* can be discerned.

Nevertheless, although not yet formally proven, it seems likely that UxpA follows the same secretion pathway as UxpB and that recognition by the Lol system needs to be avoided. Recognition by the Lol system requires full acylation of the lipoprotein with a diacylglycerol moiety attached to the sulfhydryl group of the N-terminal cysteine and an additional acyl chain attached to the α -amino group. Inhibition of the latter modification, which is catalysed by the Lnt enzyme, could retain the lipoprotein at the cytoplasmic membrane (Silva-Herzog *et al.*, 2008). Since folding of Tat substrates occurs prior to their export in the cytoplasm, one possibility is that the target site for Lnt in UxpA is sterically shielded in the folded protein. Clearly, much still needs to be learned about lipoprotein sorting in *P. putida*.

In conclusion, we have demonstrated that *P. putida* contains a complete Xcp machinery that is active in the secretion of phosphatases under P-limiting growth conditions. The secreted enzymes appear to follow a rare pathway, which involves export via a Tat system, modification by the lipoprotein-modifying enzymes, and secretion via the T2SS, to remain, eventually, attached at the bacterial cell surface. The system offers interesting opportunities for further fundamental studies, e.g. to learn the rules for lipoprotein sorting in *P. putida*. In addition, the identification of an active secretion mechanism for folded proteins in an organism that is generally regarded as safe may offer novel opportunities for the development of cell factories for the production of enzymes and of bacterial surface-exposition vectors. The identification of the signals in the secreted proteins for recognition by the secretion machinery is an important prerequisite for such applications and is our next goal. This, however, might not

be an easy task since the signal is likely conformational rather than linear and multiple recognition signals might be required when the exoproteins progress through the secretion pathway (Filloux, 2010).

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All strains were grown overnight in Luria broth unless stated otherwise. To impose iron-limitation, the medium was supplemented with 20 mM EDDHA (Sigma). For growth under phosphate-limiting conditions, we used a medium consisting of 0.12 M Tris, 80 mM NaCl, 20 mM KCl, 20 mM NH₄Cl, 3 mM Na₂SO₄, 1 mM MgCl₂, 0.2 mM CaCl₂, 2 μ M ZnCl₂, 0.5% glucose, and 0.5% BBL trypticase peptone adjusted to pH 7.5 (Levinthal *et al.*, 1962). Where indicated, the medium contained 0.05% instead of 0.5% trypticase peptone and was supplemented with 100 μ M ammonium ferric citrate (Sigma). For high-phosphate conditions, this medium was supplemented with 10 mM KH₂PO₄. *Pseudomonas putida* was grown at 30°C, shaking at 200 r.p.m. or, when indicated, at 50 r.p.m. *Escherichia coli* strains were grown at 37°C while shaking at 200 r.p.m. Antibiotics were used at the following concentrations (in μ g ml⁻¹): for *E. coli*, ampicillin 100; gentamicin 5; kanamycin 50; streptomycin 50; for *P. putida*, tetracycline 100; gentamicin 40, kanamycin 100; nalidixic acid 25; piperacillin 75; streptomycin 150.

DNA manipulations

Recombinant DNA methods were performed as described (Sambrook *et al.*, 1989) using the *E. coli* strains TOP10F' and DH5 α for routine cloning. Plasmid DNA was isolated using the Promega plasmid isolation kit according to the

Table 1. Strains used including relevant characteristics and References.

Strain	Characteristics ^a	Reference
<i>E. coli</i>		
DH5 α	F ⁻ , ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 <i>recA1 endA1 hsdR17</i> (T ₁ ⁻ , m ₁ ⁻) <i>phoA supE44 thi-1 gyrA96 relA1</i>	Invitrogen
TOP10F'	F'(<i>lacI</i> Tn10 (Tet ^R)), <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80lacZ Δ M15 Δ lacX74 <i>deoR recA1 araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Sm ^R) <i>endA1 nupG</i>	Invitrogen
CC118 λ pir	<i>araD</i> Δ (<i>ara, leu</i>) Δ lacZ74 <i>phoA20 galK thi-1 rpsE rpoB argE recA1, λpir</i>	Herrero <i>et al.</i> (1990)
<i>P. putida</i>		
KT2440	Wild-type, Nx ^R	Nelson <i>et al.</i> (2002)
KT Δ xcpS::gm	KT2440 lacking <i>xcpS</i> , Gm ^R	This study
KT Δ tat1::gm	KT2440 lacking all <i>tat-1</i> genes, Gm ^R	This study
KT Δ tat2::gm	KT2440 lacking all <i>tat-2</i> genes, Gm ^R	This study
KT Δ xcpS	KT2440 lacking <i>xcpS</i> , Gm ^S	This study
KT Δ tat1	KT2440 lacking all <i>tat-1</i> genes, Gm ^S	This study
KT Δ tat2	KT2440 lacking all <i>tat-2</i> genes, Gm ^S	This study
KT Δ xcpX::km	KT2440, <i>xcpX</i> ::mini-Tn5Km1	PRCC
WCS358	Wild-type isolate, Nx ^R	Geels and Schippers (1983)
CE1430	WCS358 Δ xcpRST, Km ^R	de Groot <i>et al.</i> (1996)

a. Tet^R, Gm^R, Km^R, Sm^R, and Nx^R indicate resistance to tetracycline, gentamicin, kanamycin, streptomycin, and nalidixic acid respectively. Gm^S, sensitive to gentamicin.

PRCC, *Pseudomonas* Reference Culture Collection.

Table 2. Plasmids used in this study and their relevant characteristics.

Plasmid	Characteristics ^a	Source or reference
pMP220	Tc ^R , IncP, promoterless <i>lacZ</i>	Spaink <i>et al.</i> (1987)
pAG112	<i>uxpA-lacZ</i> transcriptional fusion in pMP220	de Groot <i>et al.</i> (1999)
pBBR1MCS-5	Gm ^R , broad host-range cloning vector,	Kovach <i>et al.</i> (1995)
pUxpBHis	pBBR1MCS-5 encoding C-terminally his-tagged UxpB, Gm ^R	This study
pUxpBHis'	pUxpBHis, HindIII site filled in with Klenow	This study
pPS856	Amp ^R Gm ^R ; contains Gm ^R cassette flanked by <i>FRT</i> sites	Hoang <i>et al.</i> (1998)
pFLP ₂	Amp ^R ; encodes Flp recombinase	Hoang <i>et al.</i> (1998)
pCRII-TOPO	Amp ^R , Km ^R , TOPO TA cloning vector	Invitrogen
pMUTS	pCRII-TOPO with DNA segments flanking <i>xcpS</i> separated by a Gm ^R cassette ($\Delta xcpS::gm$)	This study
pMUTT1	pCRII-TOPO with DNA segments flanking the <i>tat-1</i> operon separated by a Gm ^R cassette ($\Delta tat-1::gm$)	This study
pMUTT2	pCRII-TOPO with DNA segments flanking the <i>tat-2</i> operon separated by a Gm ^R cassette ($\Delta tat-2::gm$)	This study
pKNG101	Sm ^R , R6K replicon, Mob ⁺ , <i>sacBR</i>	Kaniga <i>et al.</i> (1991)
pKFPXS	pKNG101 carrying $\Delta xcpS::gm$	This study
pKFPT1	pKNG101 carrying $\Delta tat-1::gm$	This study
pKFPT2	pKNG101 carrying $\Delta tat-2::gm$	This study
PRK2013	Km ^R , <i>ori</i> ColE1, Tra ⁺ Mob ⁺	Figurski and Helinski (1979)
PRK2073	Sm ^R , Tp ^R , pRK2013 with Tn7 inserted in Km ^R	Leong <i>et al.</i> (1982)

a. Tc^R, Gm^R, Amp^R, Km^R, Sm^R, and Tp^R indicate resistance to tetracycline, gentamicin, ampicillin, kanamycin, streptomycin, and trimethoprim respectively.

manufacturer's protocol. DNA fragments were purified from agarose gels using the Promega gel extraction kit according to the manufacturer's protocol. Plasmids were introduced in *E. coli* by transformation using the CaCl₂ procedure (Sambrook *et al.*, 1989) and into *P. putida* by tri-parental mating using the conjugative properties of plasmid PRK2013 or its derivative PRK2073. Alternatively, plasmids were introduced by electroporation (Enderle and Farwell, 1998), in 0.2 cm cuvettes at 2.5 kV, 25 μ F and 200 Ω for *E. coli* or 600 Ω for *P. putida*.

Plasmid and strain constructions

The plasmids and primers used are listed in Tables 2 and S1 respectively. For PCR amplification of chromosomal DNA fragments, a small amount of overnight grown KT2440 cells was boiled for 10 min in water, and the supernatant obtained after pelleting the cell debris in an Eppendorf centrifuge was used as template DNA. To create a plasmid encoding a His-tagged UxpB, the *uxpB* gene was amplified with primers *uxpB5B* and *uxpB3X*. The PCR product obtained was digested with BamHI and XbaI, cloned into pBBR1MC-5 digested with the same enzymes, and the resulting construct was designated pUxpBHis. In the resulting construct, the recombinant *uxpB* gene is fused in frame to an upstream located ATG initiation codon, occasionally leading to the detection of an N-terminally extended protein. To eliminate the synthesis of this product, pUxpBHis was digested at the HindIII site located between the upstream ATG and the ATG initiation codon of *uxpB*, the ends were filled in with Klenow fragment of DNA polymerase, and the plasmid was re-ligated. The resulting plasmid is dubbed pUxpBHis'.

To generate *xcpS*, *tat-1* and *tat-2* mutant derivatives of *P. putida* KT2440, we employed the Flp-*FRT* recombination system for generating markerless mutants described by Hoang and colleagues (1998). First, DNA fragments of 500 bp in length flanking the *xcpS* gene and the *tat-1* and *tat-2*

operons were amplified using the primer pairs UpFXS/UpRXS, UpFTAT1/UpRTAT1 and UpFTAT2/UpRTAT2, respectively, for the upstream regions and DnFXS/DnRXS, DnFTAT1/DnRTAT1 and DnFTAT2/DnRTAT2, respectively, for the downstream regions. A gentamicin-resistance cassette flanked by Flp recombinase target (*FRT*) sites was amplified using the primers Gm-F and Gm-R and pPS856 as the template. For each gene/operon, the upstream and downstream fragments were combined with the gentamicin-resistance cassette in between into a single DNA fragment by overlap extension PCR (Horton *et al.*, 1989). The complete fragments were then cloned into pCRII-TOPO according to the manufacturer's protocol (Invitrogen). Sequence analysis was performed to check the constructs, which were designated pMUTS, pMUTT1, and pMUTT2 respectively. The relevant fragments were then digested from these plasmids with BamHI, ligated into the suicide vector pKNG101 using the T4 DNA ligase and cloned into strain CC118 λ pir. The resulting constructs, designated pKFPXS, pKFPT2, and pKFPT2, respectively, were transferred to *P. putida* strain KT2440 by tri-parental mating, selecting for gentamicin-resistant colonies and using nalidixic acid for counter selection against *E. coli*. Recombinants obtained had the entire plasmid integrated into the chromosome as evidenced by concomitant resistance to streptomycin and sensitivity to sucrose. Double cross-overs, which had lost the plasmid sequences, were selected on plates containing 5% sucrose and designated KT $\Delta xcpS::gm$, KT $\Delta tat-1::gm$, and KT $\Delta tat-2::gm$ respectively. Loss of streptomycin resistance of the strains was confirmed. Loss of the gentamicin-resistance cassette was subsequently induced by introducing plasmid pFLP₂, encoding the Flp recombinase, via tri-parental mating selecting for transconjugants resistant to piperacillin and nalidixic acid. Transconjugants obtained were tested for sensitivity to gentamicin and further confirmed for the loss of the gentamicin-resistance cassette and the target genes by PCR. The markerless *xcpS*, *tat-1* and *tat-2* mutant

strains obtained were designated KTΔ*xcpS*, KTΔ*tat-1*, and KTΔ*tat-2* respectively.

Strain KT*xcpX::km* was obtained from the Pseudomonas Reference Culture Collection (Duque *et al.*, 2007) (strain collection number 4440) and was kindly provided by Dr Estrella Duque (CSIC-EEZ, Granada, Spain). PCR amplification of the mutant allele from chromosomal DNA and sequencing of the product confirmed the mutation and revealed that the mini-Tn5 transposon was inserted after codon 45 in the *xcpX* gene.

Enzyme assays

For β-galactosidase assays, strains were grown overnight at 30°C in P-limiting medium, either supplemented or not with 10 mM KH₂PO₄, and β-galactosidase assays were performed with *ortho*-nitrophenyl-β-galactoside as the substrate as described (Miller, 1972).

Alkaline phosphatase activities were determined using *para*-nitrophenyl phosphate (pNPP) as the substrate as described (Tomassen and Lugtenberg, 1980) with the following modifications: (i) the concentration of Tris was 0.3 M, (ii) a salt solution consisting of 40 mM NaCl, 10 mM KCl, 10 mM NH₄Cl, 1.5 mM Na₂SO₄, 0.5 mM MgCl₂, 0.1 mM CaCl₂, and 1 μM ZnCl₂ (final concentrations) was included in the reaction mixture, and (iii) reactions were performed at 30°C.

qRT-PCR

For qRT-PCR, cells from duplicate overnight cultures of strain KT2440 in LB medium were inoculated 1:20 into phosphate-limiting medium that was either supplemented or not with 10 mM KH₂PO₄. The cultures were grown at 30°C under shaking conditions until an optical density at 660 nm (OD₆₆₀) of ~1.5 was reached. Induction of the *pho* regulon in the low-P_i cultures was confirmed in an alkaline-phosphatase-activity assay. Total RNA was isolated from the bacteria using TRIzol (Invitrogen) and further purified with nucleospin RNA II columns (Macherey-Nagel). The RNA was treated with DNA-Free (Ambion) to yield DNA-free RNA and subsequently cDNA was generated from 1 μg of RNA using the Transcriptor High Fidelity cDNA synthesis kit (Roche). qRT-PCR was performed using the 7900HT Fast Real-Time PCR System and SYBR green master mix (Applied Biosystems). The primers used are listed in Table S1. Data analysis was performed using the comparative cycle threshold method (Applied Biosystems) to determine relative expression levels. 16S RNA transcript levels were used to normalize all data.

Utilization of various phosphate sources

To determine the capacity of strains to use various phosphate sources, a synthetic medium was used similar to the phosphate-limiting medium described above, but without trypticase peptone (which is a source of phosphate) added. This phosphate-free medium was solidified with 1.25% agarose. Bacterial cells were applied in a top layer of the same medium solidified with 0.5% agarose. Then, sterile filter discs (Ø 5 mm) containing 5 μl of stock solutions of different

phosphate sources were placed on top of the plate. The stock solutions contained 100 mM 5'-AMP, 20% glucose 6-phosphate, 41 mM KH₂PO₄, 25 mg ml⁻¹ XP, or 20% UDP-glucose. The plates were incubated at 30°C and growth around the filter discs was assessed every day for 3–4 days.

SDS-PAGE and Western blotting

For analysis of whole cell lysates, bacterial cells were disrupted by sonication. Whole cell lysates or extracellular protein samples were suspended in SDS-PAGE sample buffer (2% SDS, 5% DTT, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris-HCl pH 6.8) or, for semi-native SDS-PAGE, in the sample buffer without DTT. For regular SDS-PAGE, the samples were heated at 95°C for 5 min and separated on SDS-PAGE gels (Laemmli, 1970) containing 10% polyacrylamide. For semi-native SDS-PAGE, the samples were left unheated, the gels contained no SDS, and the electrophoresis was carried out on ice at 20 mA. Proteins were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes by Western blotting for immunodetection. The primary antibody used was the penta-His antibody (Qiagen) at 1:2000 dilution for a period of 1–1.5 h. Goat anti-mouse immunoglobulin G antibodies conjugated with peroxidase or alkaline phosphatase (Biosource international) were used as secondary antibodies for a period of 1 h. Subsequently, peroxidase activity was detected by chemiluminescence (Amersham, GE healthcare), while alkaline phosphatase activity was detected with 0.5 mg ml⁻¹ XP and 0.1 mg ml⁻¹ nitroblue tetrazolium (Sigma Aldrich) in 100 mM NaHCO₃, 1 mM MgCl₂ (pH 9.8).

Zymography

For zymography, semi-native SDS-PAGE was used. After electrophoresis, the gels were washed twice in 100 mM Tris-HCl, pH 8.8, 1% bovine serum albumin, after which they were incubated in reaction solution [50 mM Tris-HCl (pH 8.8), 80 mM NaCl, 20 mM KCl, 20 mM NH₄Cl, 3 mM Na₂SO₄, 1 mM MgCl₂, 0.2 mM CaCl₂, 2 μM ZnCl₂, 125 μg ml⁻¹ XP; 125 μg ml⁻¹ nitroblue tetrazolium (Sigma)] until bands became visible. The reaction was stopped by washing the gels in water.

Cell fractionation

Inner and outer membranes were separated by sucrose density gradient centrifugation as described (Hancock and Nikaido, 1978) with the following modifications: (i) DTT was added after disruption of the cells to a final concentration of 0.2 mM and was present throughout the remaining part of the procedure, (ii) isolation of the total membrane fraction on a 70% sucrose cushion was omitted, and (iii) sucrose gradient centrifugation was carried out in a Beckman SW28 rotor at 25 000 r.p.m. for 16 h. NADH-oxidase activity was determined as described (Osborn *et al.*, 1972) and the presence of OprF in the different fractions was evaluated by SDS-PAGE.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Results of conserved-domain search for UxpA and UxpB at NCBI.

Table S1. Primers used.